Effects of an extension of the equilibration period up to 96 hours on the characteristics of cryopreserved bull semen

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Abstract: This study was designed to investigate the effects of an equilibration period up to 96 hours and three extenders (AndroMed, OPTIXcell, and Triladyl) on the quality of cryopreserved bull semen and to evaluate, whether an extension of the equilibration time to 72 hours does affect fertility in the field. One ejaculate of 17 bulls was collected and divided into three equal aliquots and diluted, respectively, with the three extenders. Each aliquot was again divided into five parts and equilibrated for 4, 24, 48, 72, and 96 hours before freezing in an automatic freezer. Sperm motility, plasma membrane and acrosome integrity (PMAI), and DNA fragmentation index (% DFI) were measured during equilibration. In addition to the parameters measured during equilibration, the percentage of viable sperm cells with high mitochondrial membrane potential (HMMP) was measured immediately after thawing, and after 3 hours of incubation at 37 °C. Sperm motility was assessed using CASA, and PMAI, HMMP, and % DFI were measured using flow cytometry. Equilibration time did affect all parameters before freezing ($P < 0.01$), and also the extender affected all parameters except HMMP ($P < 0.05$). After thawing, all parameters except HMMP immediately after thawing were influenced by the equilibration period ($P < 0.001$), whereas all parameters except % DFI immediately after thawing were influenced by the extender ($P < 0.001$). The changes of semen characteristics during 3 hours of incubation were also dependent on the equilibration time and the extender used in all parameters ($P < 0.01$). In the field study, semen of nine bulls was collected thrice weekly, processed using Triladyl egg yolk extender, and frozen in 0.25 mL straws with 15 × 106 spermatozoa per straw. In total, the nonreturn rates on Day 90 after insemination (NRR90) of 263,816 inseminations in two periods were evaluated. Whereas semen collected on Mondays and Wednesdays was equilibrated for 24 hours in both periods, semen collected on Fridays was equilibrated for 4 hours in period one and equilibrated for 72 hours in period 2. No differences in NRR90 could be found ($P > 0.05$). In conclusion, extension of the equilibration time from 4 hours to 24-72 hours can improve motility and viability of cryopreserved semen after thawing. The extent of improvement in semen quality is dependent on the extender used. Prolongation of the equilibration period from 4 hours to 72 hours had no effect on fertility in the field.

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Effects of an extension of the equilibration period up to 96 hours on the characteristics of cryopreserved bull semen

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Abstract

This study was designed to investigate the effects of an equilibration period up to 96 h and three extenders (AndroMed®, OPTIXcell® and Triladyl®) on the quality of cryopreserved bull semen and to evaluate, whether an extension of the equilibration time to 72 h does affect fertility in the field. One ejaculate of 17 bulls was collected and divided into three equal aliquots and diluted, respectively, with the three extenders. Each aliquot was again divided into five parts and equilibrated for 4, 24, 48, 72 and 96 h before freezing in an automatic freezer. Sperm motility, plasma membrane and acrosome integrity (PMAI) and DNA fragmentation index (% DFI) were measured during equilibration. In addition to the parameters measured during equilibration, the percentage of viable sperm cells with high mitochondrial membrane potential (HMMP) was measured immediately after thawing, and after 3 h of incubation at 37°C. Sperm motility was assessed using CASA, and PMAI, HMMP

and % DFI were measured using flow cytometry. Equilibration time did affect all parameters before freezing ($P < 0.01$), and also extender affected all parameters except HMMP ($P < 0.05$). After thawing, all parameters except HMMP immediately after thawing were influenced by the equilibration period ($P < 0.001$), while all parameters except % DFI immediately after thawing were influenced by the extender ($P < 0.001$). The changes of semen characteristics during 3 h of incubation were also dependent on the equilibration time and the extender used in all parameters ($P < 0.01$). In the field study, semen of 9 bulls, was collected thrice weekly, processed using Triladyl® egg yolk extender and frozen in 0.25 mL straws with 15 x 10^6 spermatozoa per straw. In total, the non-return rates on day 90 after insemination (NRR90) of 263'816 inseminations in two periods were evaluated. While semen collected on Mondays and Wednesdays was equilibrated for 24 h in both periods, semen collected on Fridays was equilibrated for 4 h in Period 1 and equilibrated for 72 h in Period 2. No differences in NRR90 could be found ($P > 0.05$). In conclusion, extension of the equilibration time from 4 h to 24-72 h can improve motility and viability of cryopreserved semen after thawing. The extent of improvement in semen quality is dependent on the extender used. Prolongation of the equilibration period from 4 h to 72 h had no effect on fertility in the field.

Keywords:
Bull semen
Cryopreservation
Equilibration period
Extender
Semen quality
Fertility

1. Introduction

During the development of bovine semen freezing technology each step between semen collection and freezing has been carefully evaluated, including duration of equilibration. In a review, Pickett and Berndtson [1] deduce from a series of fertility trials that slow cooling and equilibration at 5 °C are important for optimal fertility and recommend an equilibration time of 4 to 18 hours. The equilibration time is thought to be important for sperm membranes to adapt to low temperatures [2, 3] and to enable the translocation of water, hence decreasing the damage by ice nucleation during freezing-thawing [4].

There is no agreement on what time of equilibration is best for semen quality after cryopreservation but there is a desire to control this step in order to optimize the production line in commercial AI centers. In recent literature there is a wide range of equilibration periods reported: no equilibration at all [3, 5], 30 min [6], 1.5 to 4 h [3, 5, 7–10], 18 to 28 h [10–12]. In experiments where it was examined, whether an equilibration period is necessary at all, 2 h [5] or 4 h of equilibration [3] delivered better results than cryopreservation without equilibration. Therefore the equilibration period seems to be necessary for good semen quality after cryopreservation. When an equilibration period of 3 to 4 hours was compared to equilibration overnight (18 or 24 h) there was a higher post-thaw motility with the longer equilibration period [10, 11], although no difference in fertility could be found when comparing 4 and 28 h of equilibration period [11].

Muiño et al. [2] evaluated extenders with and without egg yolk using a prolonged equilibration time of 18 h and found higher sperm survival and longevity for the egg yolk-containing extender. However there is few data in literature about the effect of different extenders, with and without egg yolk, using a prolonged equilibration period.

Currently, there is a trend against using animal products in extenders because of hygienic risks, the lack of quality standards and the presence of steroid hormones, which may reduce the fertilizing capacity of spermatozoa [2]. Substances of animal origin represent a risk for microbial contamination with the subsequent production of endotoxins capable of damaging the fertilizing capacity of spermatozoa [13].

With the aim to optimize the production line of a commercial AI center and improve the quality of semen collected and frozen on Fridays, we examined, whether it is possible to prolong the equilibration time up to 96 h, using extenders with and without animal products without compromising sperm quality and fertility.

2. Materials and methods

2.1 Semen collection and processing

A total of 17 bulls (Brown Swiss n = 7, Red Holstein n = 7, Limousin n = 2 and Holstein Friesian n = 1), aged between 18 and 36 months were used for the experiment. One ejaculate of each bull fulfilling minimum standards of progressive motility (70%) and sperm concentration (500 x 10^6/mL) was processed with three different extenders to obtain a final sperm concentration of 60 x 10^6 spermatozoa per mL. The extenders used were Triladyl® (Minitube, Tiefenbach, Germany) a TRIS-egg yolk based extender, and the two extenders AndroMed® (Minitube, Tiefenbach, Germany) and OPTIXcell® (imv, L'Aîgle, France) containing no animal originating substances. Each aliquot was once again divided into 5 parts and equilibrated at 4 °C for 4, 24, 48, 72 and 96 h before packaging at 4 °C in 0.5 mL straws with a concentration of 60 x 10^6 spermatozoa per mL. Thereafter the straws were frozen in an automatic freezer (Microdigitcool, imv, L'Aîgle, France) and stored in liquid nitrogen at -196 °C.

104 2.2 Semen laboratory analysis

105 Semen characteristics were assessed after equilibration times of 4, 24, 48, 72 and
106 96 h as well as after freezing-thawing and pooling the contents of 3 straws
107 immediately (0 h) and after additional 3 h of incubation at 37 °C.

108

109 2.2.1 Sperm motility assessment with CASA

110 The IVOS II CASA system driven by software version 14 (Hamilton Thorne Inc.,
111 Beverly, U.S.A.) was used to assess sperm motility. For the measurements equal
112 parts of extended semen and of Tyrode's solution were mixed and analyzed after
113 10 min (0 h) and 3 h of incubation at 37 °C. To semen extended with egg yolk-free
114 extenders equal parts of Tyrode were added and measured after additional 10 min of
115 incubation at 37 °C. In semen extended with Triladyl® Tyrode's solution containing
116 80 µg/mL Hoechst 33342 was used to stain sperm DNA in order to discriminate
117 accurately between sperm and nonsperm particles (especially egg yolk components)
118 [14] using the Ident Fluorescence Option "Full Analysis" of the IVOS II system. For
119 each sample, a 20 µm-deep semen analysis Leja 4-chamber slide (Leja, Nieuw-
120 Vennep, the Netherlands) placed on a pre-warmed stage (37 °C) was filled with
121 semen and a minimum of 1000 cells were analyzed in no less than eight randomly
122 selected fields, with 30 frames acquired per field at a frame rate of 60 Hz. For further
123 analysis the percentage of rapid cells with Average Path Velocity (VAP) ≥50 µm/s
124 was considered as sperm motility.

125

126 2.2.2 Chemicals and reagents

127 Chemicals used for the preparation of Tyrode's solution, TNE buffer (0.01 M Tris,
128 0.15 M NaCl, 1 mM EDTA, pH 7.4) and acridine orange (AO) staining buffer (0.2 M
129 Na2HPO4, 1 mM EDTA, 0.15 M NaCl, 0.1 M citric acid, pH 6.0), as well as the

fluorescein isothiocyanate-conjugated lectin from *Arachis hypogaea* (FITC-PNA), propidium iodide (PI), Hoechst 33342 and Triton-X were purchased from Sigma-Aldrich Co. (Buchs, Switzerland). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) was obtained from Life Technologies Europe B.V. (Zug, Switzerland), while AO was purchased from Polysciences Europe GmbH (Eppelheim, Germany). Fluorescent probes were diluted and used for sperm staining in form of working solutions with following concentrations: 2.99 mM PI, 100 µg/mL FITC-PNA and 0.153 mM JC-1.

2.2.3 Plasma membrane and acrosome integrity and mitochondrial membrane potential of sperm

Flow cytometric assays regarding plasma membrane and acrosome integrity as well as mitochondrial membrane potential were performed using a Cell Lab Quanta SC MPL flow cytometer, operated by the Cell Lab Quanta SC Software for Instrument Control Data Acquisition (Beckman Coulter Inc., Nyon, Switzerland), which was equipped with a solid state laser exciting at 488 nm and emission filters detecting green, orange and red fluorescence at 525, 590 and 670 nm, respectively. Flow rate was set to 500 cells/s and for each sample 10’000 events were analyzed. The Cell Lab Quanta SC Software for Instrument Control Data Analysis (Beckman Coulter Inc., Nyon, Switzerland) was used for cell gating and data analysis.

For the assessment of plasma membrane and acrosome integrity (PMAI), 5 µL of semen, previously diluted in 241 µL of Tyrode’s solution, were stained with 1.5 and 2.5 µL of PI and FITC-PNA and flow cytometrically assessed after 15 min incubation at 37 °C. After gating out non-cellular events, the percentage of PI- and FITC-PNA-negative cells, with intact plasma membrane and acrosome (PMAI, %) was determined.

To determine the percentage of viable sperm cells with high mitochondrial membrane potential (HMMP, %), 1.5 µL PI and 2.5 µL JC-1 were added to 5 µL of sperm, previously diluted in 241 µL of Tyrode's solution and samples were analyzed after 15 min incubation (37 °C). After gating out PI-positive cells, the percentage of viable sperm showing high mitochondrial membrane potential (HMMP) was identified.

2.2.4 DNA fragmentation

The Sperm Chromatin Structure Assay (SCSA®) was performed to assess sperm DNA integrity, using a Coulter EPICS XL flow cytometer driven by EXPO32 ADC XL 4 Color™ software (Beckman Coulter Inc., Krefeld, Germany). Cells were excited by a 488 nm Argon laser and the emitted green, orange or red fluorescence was captured at 525, 575 or 620 nm, respectively. A total of 10'000 events were analyzed for each sample at a flow rate of 200 cells/s. Data analysis and computation of SCSA parameters were performed using the 4.07.0005 version of FCS EXPRESS Flow Cytometry Research Edition software (De Novo Software, Glendale, USA).

The percentage of cells with high DNA fragmentation index (% DFI) was assessed performing the SCSA™ [15]. In short, 400 µL of acid detergent solution (0.15 M NaCl, 0.08 N HCl, 0.1% Triton-X 100, pH 1.2) were added to 200 µL of semen previously diluted with TNE buffer to a final concentration of 1-2 $x10^6$ sperm/mL. Following thorough mixing of the sample and 30-second incubation, 1.2 mL of AO staining solution (6.0 µg AO/mL AO staining buffer) were added and stained samples were flow cytometrically assessed after exactly 3 min. Cell gating and quantification of the percentage of cells with high DNA fragmentation index (% DFI) were performed as previously described by Evenson and Jost (2001).

2.3 Field study

To evaluate the effect of different equilibration times on fertility, the non-return rates on day 90 after insemination (NRR90) of 263'816 artificial inseminations were evaluated. Ejaculates (n = 2'456) were collected thrice weekly (Monday, Wednesday, Friday) from 9 Simmental bulls aged between 3 and 9 years on a commercial artificial insemination center (Besamungsverein, Neustadt a.d. Aisch, Germany) and processed using Triladyl® egg yolk extender to obtain a final sperm concentration of 60 x 10^6 spermatozoa per mL and 15 x 10^6 spermatozoa per straw. For NRR90 cows were assumed to be pregnant if a subsequent insemination was not reported within 90 days after the initial breeding. Data were collected from all inseminations performed with straws from the recorded ejaculates between January 2011 and May 2015. In Period 1, ejaculates collected on Mondays and Wednesdays were processed with an equilibration time of 24 h, on Fridays the equilibration was 4 h. In Period 2, the equilibration time of ejaculates collected on Mondays and Wednesdays were 24 h and the ejaculates collected on Fridays were equilibrated for 72 h before freezing (see Table 1).

2.4 Statistical analysis

The data were analyzed using R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria) version 3.2.3 and the software package lmtest [16]. The experiment was analysed as a two-way factorial split experiment. The variables motility, PMAI, and % DFI during equilibration as well as motility, PMAI, % DFI and HMMP immediately after thawing and after additional 3 h of incubation at 37 °C were analyzed with a GLM (general linear model) with the factors extender, equilibration time and their interaction terms. P-values were derived by post-hoc pairwise comparisons with a Tukey's test and considered as significant when < 0.05. All values are presented as percentages. The

rates of change in post-thaw semen characteristics during 3 h of incubation at 37 °C in percent were calculated as follows: (*value at 3 h – value at 0 h*) / *value at 0 h* x 100. The overall effect of extender, equilibration time and their interaction term on the rate of change of motile sperm, PMAI, % DFI and HMMP between 0 h and 3 h were analysed using a mixed-effect linear model, with bull as random effect and extender, equilibration and the interaction term as fixed effects. Pairwise comparisons between levels of the factor equilibration time were assessed with planned (a priori) polynomial contrasts.

Mean NRR per bull and weekday of semen collection were evaluated using an analysis of variance with the predicting variable weekday of semen collection with the Bonferroni correction method for posthoc pairwise comparisons.

3. Results

3.1 Semen characteristics during equilibration before freezing

Equilibration time did affect all parameters measured before freezing ($P < 0.01$; Table 2). All parameters deteriorated with time, reaching within 48 h inferior values ($P < 0.05$) compared to 4 h of equilibration. Additionally, extender had an impact on motility and PMAI ($P < 0.05$). The use of Triladyl® resulted in higher overall values for motility than OPTIXcell® ($P < 0.01$), which in turn was superior to AndroMed® ($P < 0.01$). For PMAI there was no difference between Triladyl® and OPTIXcell®, which both had higher overall values than AndroMed® ($P < 0.01$ and $P < 0.05$).

3.2 Semen characteristics after freezing and thawing

Equilibration time had an effect ($P < 0.001$) on all parameters measured immediately after freezing-thawing except on HMMP. After additional 3 h of incubation at 37 °C, all parameters were affected by equilibration time ($P < 0.001$). Values of all sperm

234 parameters except % DFI determined immediately after thawing depended on the
235 type of extender (Table 3). Motility for 0 h and 3 h after thawing was highest after
236 24 h of equilibration (Fig. 1). Triladyl® and OPTIXcell® performed similarly ($P > 0.05$),
237 while AndroMed® showed lower values ($P < 0.001$), especially after 48 h of
238 equilibration. Values for PMAI for both 0 h and 3 h were higher after 24 h compared
239 to 4 h of equilibration ($P < 0.05$) and deteriorated with time, being lower than at 24 h
240 by 96 h at the latest ($P < 0.001$). Triladyl® and OPTIXcell® gave similar ($P > 0.05$)
241 results for 0 h, while AndroMed® had lower values ($P < 0.001$; Fig. 2). After 3 h of
242 incubation PMAI was higher in frozen-thawed sperm extended in OPTIXcell® than in
243 sperm cryopreserved with Triladyl® ($P < 0.001$), which in turn had higher values than
244 sperm processed with AndroMed® ($P < 0.001$). HMMP immediately after freezing did
245 not change ($P > 0.05$) with the duration of equilibration time, while after 3 h of
246 incubation values for equilibration times of 48 h or more resulted in lower values than
247 4 h and 24 h ($P < 0.001$). Sperm equilibrated with OPTIXcell® resulted in higher
248 HMMP values at 0 h and 3 h after freezing than AndroMed®, and Triladyl® ($P < 0.05$
249 and $P < 0.001$). Overall values for % DFI after 0 h and 3 h after freezing were not
250 different between 4 h and 24 h of equilibration time, but reached lower values within
251 96 h ($P < 0.01$) when compared to 24 h of equilibration. All extenders gave similar
252 overall results for % DFI immediately after freezing, however, after 3 h of incubation
253 at 37 °C, Triladyl® had higher values than the other extenders ($P < 0.01$), especially
254 for 72 h and 96 h of equilibration.
255
256 3.3 Change of semen characteristics between 0 h and 3 h after thawing
257 The extender used and the length of the equilibration time had an effect ($P < 0.01$) on
258 the percent change between 0 h and 3 h after freezing-thawing in all semen
259 characteristics analysed (Table 3). No differences in the percent changes between

the equilibration times 4 h and 24 h could be found in any parameter ($P > 0.05$). With increasing equilibration time the percent changes increased, being higher ($P < 0.05$) by 96 h of equilibration at the latest when compared to 24 h. For motility and PMAI, OPTIXcell® showed the smallest decreases. While Triladyl® had a smaller decrease in motility than AndroMed®, it was the other way round for PMAI. Percent change in HMMP was smallest for OPTIXcell® followed by AndroMed® ($P < 0.001$) and Triladyl® ($P < 0.05$). No differences between OPTIXcell® and AndroMed® could be found for % DFI for any equilibration time and while Triladyl® was on a comparable level at 4 h, percent changes were higher from 24 h on ($P < 0.05$).

3.4 Field study

There were no differences in NRR90 ($P > 0.05$) neither within production periods between weekdays nor between production periods (Table 4).

4. Discussion

The findings of this study show that bull semen can be equilibrated at 4 °C for up to 72 h without compromising sperm quality and fertility. However, sperm quality is strongly dependent on the type of extender used.

Semen characteristics during equilibration revealed a slow decrease of all parameters with time. Rickenbacher [10], who compared semen parameters during equilibration periods of 0 h to 24 h, could not find a difference in motility and PMAI after 4 h or 24 h of equilibration, which is consistent with our findings, since significant differences were only apparent after 48 h. The decrease with time was dependent on the extender used for the parameters motility and PMAI, with the egg yolk containing Triladyl® showing the slowest decrease, seeming to be best suited to

protecting sperm from negative effects, such as continuous exposure of spermatozoa to bovine seminal plasma (BSP) proteins during equilibration [17].

In frozen-thawed semen immediately after thawing, despite of the slow decrease of all semen characteristics during equilibration, values for motility and PMAI were higher for 24 h compared to 4 h of equilibration and remained on or above the level of 4 h until at least 72 h. This indicates the beneficial effect of a prolonged equilibration at 4 °C before freezing to obtain optimal semen quality and is in agreement with Rickenbacher et al. [10] who compared equilibration times between 1.5 h and 24 h using TRIS-egg yolk extender and found the highest values for motility and PMAI after 24 hours of equilibration. Also Foote and Kaproth [11] achieved higher motility after 18 h compared to 4 h of equilibration using a whole milk extender. Anzar et al. [12] reported higher motility and more spermatozoa with intact plasma membranes after at least 24 h of shipping at 4 °C using TRIS-egg yolk extender compared to control semen equilibrated for 2 h. But while they saw an alteration of mitochondrial function using a prolonged equilibration time, we could not see any change in HMMP with time. This difference might be explained by different definitions of the parameter HMMP. While Anzar et al. [12] expressed the percentage of sperm with intact plasma membrane and high mitochondrial potential of all sperm, we calculated the percentage of sperm with high mitochondrial membrane potential of sperm with intact plasma membrane only, thereby minimizing effects of a change in sperm viability. Comparing extenders with and without egg yolk using an equilibration time of 18 h, Muiño et al., [2] recorded higher sperm survival and acrosome integrity using the two-step TRIS-egg yolk extender Biladyl® than using two egg yolk-free extenders and attributed this effect to the better protective effects of egg yolk lipoproteins than of soybean-derived components during the equilibration at 4 °C. Previous work comparing soy bean-derived extenders with TRIS-egg yolk

312 based extenders, using equilibration periods of 2-5 h, reported inconsistent results.
313 Hinsch et al., [18] found no differences in post-thaw motility, plasma and acrosomal
314 membrane integrity and fertility of semen extended with Triladyl® or Biociphos®, [7–9,
315 18]. Others reported a decrease in post-thaw motility, viability, morphology and
316 osmotic resistance [8] or a reduction in the 56-day non-return rate [7] when bull
317 spermatozoa were cryopreserved with Biociphos Plus® compared with Tris-egg yolk
318 extenders. The use of AndroMed® resulted in higher post-thaw motility and non-
319 return rates on day 56 compared to semen extended with Tris-egg yolk [9]. In the
320 present study, the soy bean-derived extender AndroMed® yielded inferior results
321 compared to the TRIS-egg yolk extender Triladyl® for the parameters motility and
322 PMAI, while yielding higher results for HMMP. However the liposome based extender
323 OPTIXcell® showed similar values as Triladyl® for motility and PMAI, resulting in even
324 higher values for HMMP. Altogether, our results and the data in literature show that
325 the effect of an extender is strongly dependent on various factors of semen
326 processing.
327 The presumed cryoprotective mechanism of egg yolk is through sequestration of BSP
328 proteins by low-density lipoproteins, reducing the cholesterol and phospholipid efflux
329 and thus maintaining the resistance of the plasma membrane to low temperatures
330 [19, 20]. The underlying mechanisms of how liposomes stabilize cells during freezing
331 are still poorly understood. A possible explanation could be that liposomes modify
332 sperm membranes by exchanging lipids and cholesterol, thus modifying membrane
333 physical properties at reduced temperatures [21]. Consequently the lipid phase
334 transition temperature – where the membrane changes from a liquid crystalline state
335 to a gel phase – is lowered, increasing the fluidity of the sperm membrane at low
336 temperatures [22] and improving the cryostability of the cells. While cholesterol can
337 exchange rapidly between different membrane bilayers [23, 24] lipid transfer is a

relatively slow process [25], potentially explaining the positive effect of a prolonged equilibration period, especially when OPTIXcell® or Triladyl-egg yolk are used as extenders.

After 3 h of incubation at 37 °C after freezing-thawing, the results were similar as immediately after freezing with values being slightly inferior, while the differences between extenders and the variability became more pronounced. The percent change between 0 h and 3 h after freezing-thawing and its variability also increased with the duration of the equilibration period. Using an equilibration time of 4 h motility decreased 13.2 ± 1.8 % in our study, while Anzar et al., [12] only found a decrease of 1 ± 2.4 % for semen equilibrated for 2 h and incubated at 37 °C for 2 h. For semen shipped for at least 24 h at 4 °C, the decrease was larger, being closer to the values in our study (8 ± 0.7 % vs. 13.6 ± 1.8 %, respectively). Decreases in HMMP during incubation were at least 20 % in our study, while Anzar et al. [12] found no decrease after 2 h of incubation in both groups. Possible reasons for this difference include different breeds of the bulls used, different extenders and freezing protocols. The values for % DFI 3 h after freezing-thawing showed a significant raise for Triladyl® compared with the two egg yolk-free extenders, which was caused by a much larger percent change during the incubation at 37 °C (Fig. 4). Possible explanations are a lower content in antioxidants or microbial contamination of the egg yolk generating reactive oxygen species (ROS) which might have caused lipid peroxidation of spermatozoa [26] leading to disruption of its membrane conformation and loss of motility [27] resulting in oxidative DNA damage [28] increasing with equilibration time.

No difference in fertility could be seen when increasing equilibration from 4 h to 72 h, which is consistent with Foote and Kaproth [11], who compared NRR on day 56 using semen processed with whole milk extender equilibrated for 4 h to semen processed with whole milk-fructose extender equilibrated for 4 h or 28 h, respectively, and found

no difference either. A difference in semen characteristics might have been masked by the high number of spermatozoa per insemination dose used, levelling out negative effects of compensable sperm defects. In order to reliably detect differences in fertility, a large-scale low-dose insemination trial would be necessary, with the number of spermatozoa being below the maximum pregnancy rate value for individual bulls, ranging from 0.5 – 12 x 10^6 sperm per dose [29, 30].

In conclusion, the extension of the equilibration time from 4 h to 24-72 h improves motility and viability of cryopreserved semen after thawing. Optimal values for most parameters were measured after 24 h with the extent of improvement in sperm quality being dependent on the extender used. Extension of the equilibration period from 4 h to 72 h did not have an effect on fertility in the field.

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470

Table 1: Ejaculates processed, equilibration applied and inseminations performed in relation to weekday of semen collection in the two periods evaluated.

	Monday	Wednesday	Friday
Period 1			
- Equilibration (h)	24	24	4
- Ejaculates (n = 1'068)	367	384	317
- Inseminations (n = 127'157)	47'928	46'864	32'365
Period 2			
- Equilibration (h)	24	24	72
- Ejaculates (n = 1'388)	467	474	447
- Inseminations (n = 136'659)	46'695	49'297	40'667

Table 2: Mean (± SD) of sperm motility, plasma membrane and acrosome intact spermatozoa (PMAI) and DNA Fragmentation Index (% DFI) of bull semen (n = 17) processed with AndroMed®, OPTIXcell® and Triladyl® – egg yolk extender during equilibration (0-96 h).

	Equilibration					Extender		
	4 h	24 h	48 h	72 h	96 h	AndroMed®	OPTIXcell®	Triladyl®
Motility (%)	85.1±7.6^{a}	83.5±7.3^{a,b}	79.3±8.1^{b,c}	74.7±10.6^{c}	67.3±15.4^{d}	73.4±15.2^{a}	77.9±10.3^{b}	82.7±7.7^{c}
PMAI (%)	86.1±5.5^{a}	84.3±5.8^{a,b}	82.3±6.6^{b,c}	79.9±7.2^{c,d}	77.3±8.7^{d}	79.8±7.7^{a}	82.3±6.9^{b}	83.8±7.4^{b}
% DFI	3.1±1.4^{a}	3.7±1.5^{a,b}	4.2±1.6^{b,c}	4.8±1.7^{c}	5.8±2.1^{d}	4.5±2.3	4.0±1.7	4.5±1.6

^{a,b} Different letters within the same row indicate significant ($P < 0.05$) differences, related to equilibration time and extender, respectively.

Table 3: Mean (± SD) of sperm motility, plasma membrane and acrosome intact spermatozoa (PMAI), viable sperm showing high mitochondrial membrane potential (HMMP) and DNA Fragmentation Index (% DFI) of bull semen (n = 17) processed with AndroMed®, OPTIXcell® and Triladyl® – egg yolk extender using equilibration times of 0-96 h, immediately (0 h) and 3 h after freezing-thawing as well as the percent change (%Δ) between 0 h and 3 h.

	Equilibration					Extender		
	4 h	24 h	48 h	72 h	96 h	AndroMed®	OPTIXcell®	Triladyl®
Motility (%)								
- 0 h	51.0±12.4^{a}	59.6±11.7^{b}	55.4±14.3^{a,b}	50.1±14.6^{a,c}	44.0±16.8^{c}	42.1±15.6^{a}	55.9±12.7^{b}	58.1±10.9^{b}
- 3 h	44.5±13.0^{a}	51.8±13.2^{b}	44.1±15.7^{a}	35.1±16.5^{c}	25.8±16.4^{d}	30.6±17.2^{a}	45.2±12.1^{b}	45.0±18.2^{b}
- %Δ	-13.2±12.6^{a}	-13.6±12.5^{a}	-21.8±15.6^{a}	-32.8±20.7^{b}	-44.9±27.1^{c}	-32.5±23.7^{a}	-18.6±14.0^{b}	-24.7±24.8^{b}
PMAI (%)								
- 0 h	49.5±11.1^{a}	67.0±10.5^{b}	65.7±11.0^{b,c}	64.2±10.5^{b,c}	59.5±10.6^{d}	51.6±12.0^{a}	66.5±9.5^{b}	65.4±9.5^{b}
- 3 h	38.1±10.0^{a}	54.1±11.4^{b}	49.7±11.3^{b,c}	47.9±12.9^{c}	41.1±12.4^{a}	38.9±10.9^{a}	54.0±12.4^{b}	45.7±10.8^{c}
- %Δ	-22.8±10.3^{a,b}	-19.5±9.7^{a}	-24.5±10.3^{a,b}	-25.6±13.4^{b,c}	-31.3±13.2^{c}	-24.5±11.1^{a}	-19.4±11.1^{b}	-30.4±11.5^{c}
HMMP (%)								

- 0 h	91.4±6.1	91.5±7.2	91.7±4.6	92.9±9.0	91.9±8.7	92.2±6.3[a]	95.0±3.0[b]	88.4±9.4[c]
- 3 h	71.4±16.6[a]	70.2±14.7[a]	52.5±17.0[b]	52.6±21.7[b]	43.9±24.2[c]	54.2±23.3[a]	72.6±16.2[b]	47.6±17.5[c]
- %Δ	-21.9±17.3[a]	-22.9±16.6[a]	-43.0±17.6[b]	-43.9±21.9[b,c]	-52.3±24.9[c]	-40.7±2.8[a]	-23.4±1.9[b]	-46.3±2.1[a]
% DFI								
- 0 h	5.2±2.3[a]	5.8±2.5[a,b]	6.5±2.7[a,b,c]	7.0±2.8[b,c]	7.7±2.8[c]	6.5±2.7	6.1±2.4	6.7±3.1
- 3 h	6.4±2.6[a]	7.0±3.0[a,b]	8.2±3.3[b,c]	9.0±3.8[c,d]	10.6±4.5[d]	7.3±2.7[a]	7.0±2.8[a]	10.3±4.6[b]
- %Δ	25.4±27.6[a,b]	21.4±28.9[a]	27.3±33.3[a,b]	32.3±42.7[a,b]	39.9±50.1[b]	14.8±15.9[a]	15.1±12.4[a]	57.9±51.6[b]

485 [a,b] Different letters within the same row indicate significant ($P < 0.05$) differences, related to
486 equilibration time and extender, respectively.

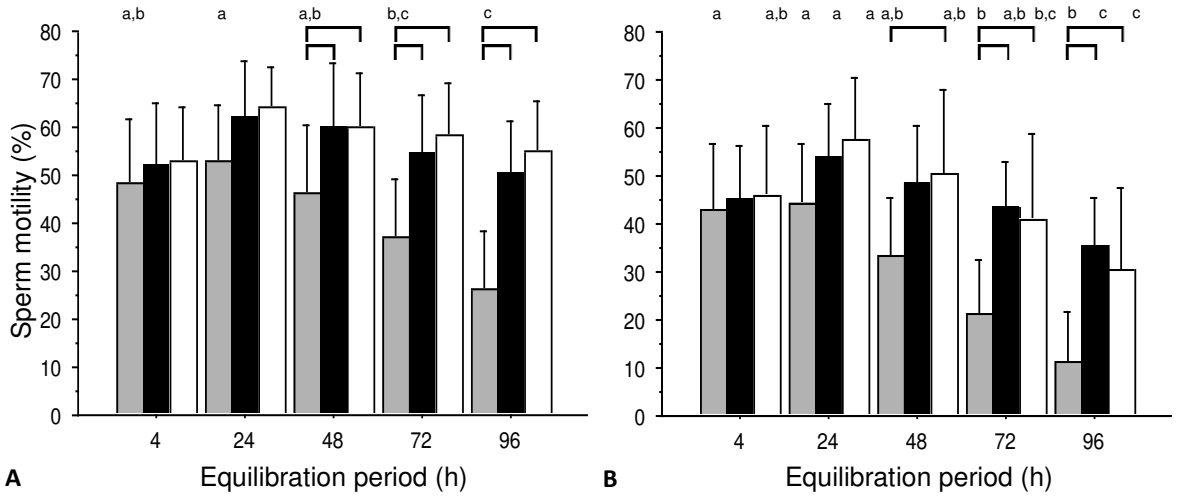
487

488 Table 4: NRR90 (mean ± SD) of semen processed on different weekdays during periods 1 and 2.

	Monday	Wednesday	Friday
Period 1	64.4±1.0	65.0±1.2	65.1±1.1
Period 2	66.0±1.0	65.6±0.9	64.7±0.8

489

490



491

492 Fig 1. Mean (± SD) of sperm motility (%) immediately after thawing (A) as well as
493 after additional 3 h of incubation at 37 °C (B) in relation to the extender used (
494 AndroMed®, ■ OPTIXcell®, □ Triladyl®) and the equilibration applied.

495 Brackets indicate significant ($P < 0.05$) differences between extenders within equilibration period.
496 [a,b] Different letters indicate significant ($P < 0.05$) differences between different equilibration periods
497 within extender.

498

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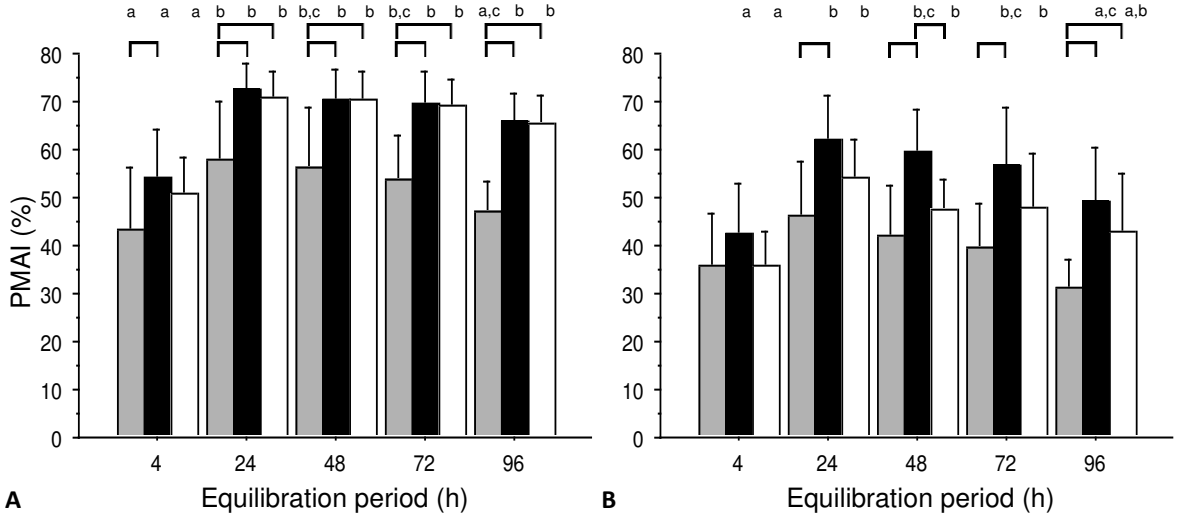


500

501 Fig 2. Mean (± SD) of plasma membrane and acrosome intact sperm (PMAI) immediately
502 after thawing (A) as well as after additional 3 h of incubation at 37 °C (B) in relation to the
503 Extender used (AndroMed®, ■ OPTIXcell®, □ Triladyl®) and the equilibration applied.

Brackets indicate significant (P < 0.05) differences between extenders within equilibration period.

[a,b] Different letters indicate significant (P < 0.05) differences between different equilibration periods within extender.

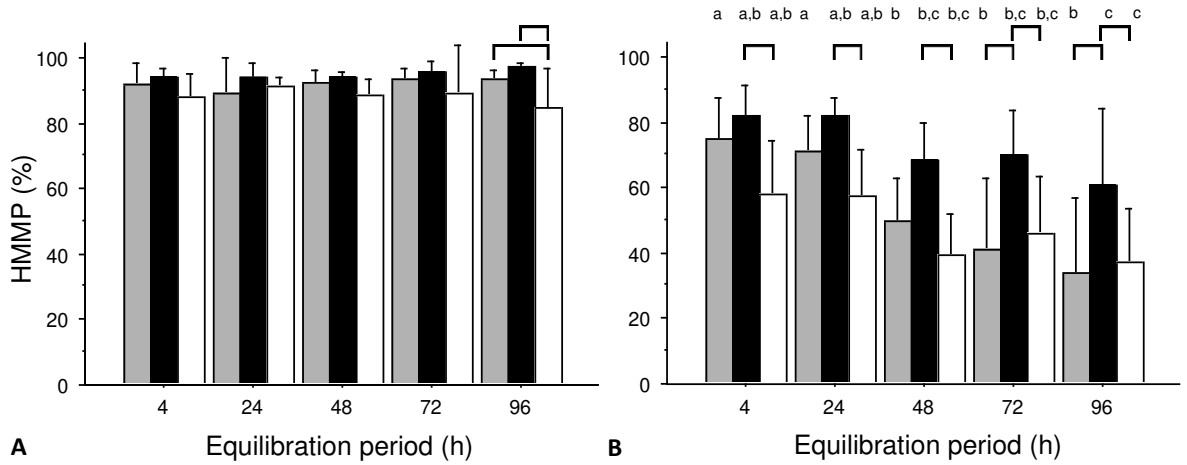
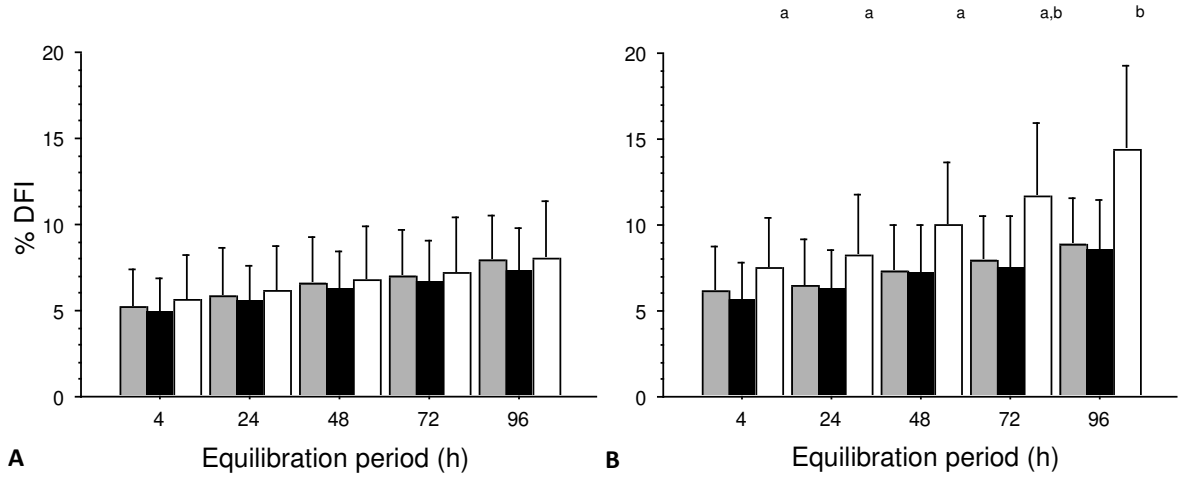


Fig 3. Mean (± SD) of viable sperm showing high mitochondrial membrane potential (HMMP) immediately after thawing (A) as well as after additional 3 h of incubation at 37 °C (B) in relation to the extender used (▒ AndroMed®, ■ OPTIXcell®, □ Triladyl®) and the equilibration applied.

Brackets indicate significant (P < 0.05) differences between extenders within equilibration period.

[a,b] Different letters indicate significant (P < 0.05) differences between different equilibration periods within extender.



519

520 Fig 4. Mean (± SD) of DNA Fragmentation Index (% DFI) immediately after thawing (A) as
521 well as after additional 3 h of incubation at 37 °C (B) in relation to the extender used
522 [AndroMed®, ■ OPTIXcell®, □ Triladyl®) and the equilibration applied.

523 Brackets indicate significant ($P < 0.05$) differences between extenders within equilibration period.
524 [a,b] Different letters indicate significant ($P < 0.05$) differences between different equilibration periods
525 within extender.